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Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins

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Abstract

Extraction of soybean seed proteins for two-dimensional polyacrylamide gel electrophoresis (2D–PAGE) and mass spectrometry analysis is challenging and inconsistent. In this study, we compared four different protein extraction/solubilization methods—urea, thiourea/urea, phenol, and a modified trichloroacetic acid (TCA)/acetone—to determine their efficacy in separating soybean seed proteins by 2D–PAGE. In all four methods, seed storage proteins were well separated by 2D–PAGE with minor variations in the intensity of the spots. The thiourea/urea and TCA methods showed higher protein resolution and spot intensity of all proteins compared with the other two methods. In addition, several less abundant and high molecular weight proteins were clearly resolved and strongly detected using the thiourea/urea and TCA methods. Protein spots obtained from the TCA method were subjected to mass spectrometry analysis to test their quality and compatibility. Fifteen protein spots were selected, digested with trypsin, and analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS) and liquid chromatography mass spectrometry (LC–MS). The proteins identified were β-conglycinin, glycinin, Kunitz trypsin inhibitor, alcohol dehydrogenase, Gly m Bd 28K allergen, and sucrose binding proteins. These results suggest that the thiourea/urea and TCA methods are efficient and reliable methods for 2D separation of soybean seed proteins and subsequent identification by mass spectrometry.
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Two-dimensional polyacrylamide gel electrophoresis (2D–PAGE)[1] is one of the most powerful proteomics tools for the separation and quantification of proteins. There have been a number of recent advances in 2D methodologies, including improved sample application and use of immobilized pH gradient (IPG) strips for isoelectric focusing (IEF), allowing more proteins to be arrayed in micropreparative quantities [1,2]. However, protein extraction techniques remain a challenge in the accurate analysis of proteins due to the presence of contaminants that affect the performance of the 2D–PAGE. Among several proteomics tools, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS) and liquid chromatography mass spectrometry (LC–MS) are sensitive methods for accurately characterizing protein profiles. This use in determining possible alterations of protein profiles

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[1] *Abbreviations used:* 2D–PAGE, two-dimensional polyacrylamide gel electrophoresis; IPG, immobilized pH gradient; IEF, isoelectric focusing; MALDI–TOF–MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; LC–MS, liquid chromatography mass spectrometry; GMO, genetically modified; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylendiamine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; CHCA, α-cyanohydroxycinnamic acid; TCA, trichloroacetic acid; NCBI, National Center for Biotechnology Information.

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present in genetically modified (GMO) soybean has become increasingly popular. Together with 2D–PAGE, these tools can be used both to visualize and compare complex mixtures of proteins and to gain a large amount of information about the individual proteins involved in specific biological responses [3–5].

Although several methods for 2D analysis of plant and seed proteins have been reported in a variety of crops [6–13], only a limited number of methods have been reported for soybean seed protein analysis [14–16]. In this study, we compared four different methods of soybean seed protein extraction for their compatibility with 2D–PAGE analysis and with respect to their efficiency in solubilizing proteins and subsequent identification by MALDI–TOF–MS and LC–MS.

Materials and methods

Chemicals

Chemicals for electrophoresis, including acrylamide, bis-acrylamide, SDS, *N*,*N*,*N*′,*N*′-tetramethylethylendiamine (TEMED), ammonium persulfate, thiourea, dithiothreitol (DTT), and CHAPS, were purchased from GE Healthcare (Piscataway, NJ, USA). Urea and ampholytes (pH 3–10) were purchased from Bio-Rad (Hercules, CA, USA). Tris–HCl (pH 8.8), ethylenediaminetetraacetic acid (EDTA), 2-mercaptoethanol, glycerol, sucrose, and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). α-Cyanohydroxycinnamic acid (CHCA) matrix was purchased from Bruker Daltonics (Billerica, MA, USA). All other chemicals were standard reagent grade laboratory chemicals. Water from a Millipore Milli-RO4 reverse osmosis system was used for making all solutions.

Plant materials

Soybean seeds [*Glycine max* (L.) Merr.] of cultivar Williams 82 were obtained from the U.S. Department of Agriculture soybean germplasm collection (Urbana, IL, USA). Seeds were stored at −80 °C until use.

Extraction buffers

In this investigation, four different extraction buffers/methods were used to extract protein from the soybean seeds.

Urea solubilization buffer

In this protocol, soybean seeds were frozen in liquid nitrogen and ground into a fine powder and solubilized according to Berkelman and coworkers [17]. Protein was extracted by vortexing 100 mg of seed powder with 300 µl of lysis solution (8 M urea, 4% CHAPS, 2% ampholyte [pH 3–10]) and was sonicated for 40 min at room temperature. The extract was centrifuged at 20,800*g* for 10 min. The supernatant was further cleaned using a 2D cleanup kit according to the manufacturer's instructions (GE Healthcare), and an aliquot was used to determine the concentration of protein.

Thiourea/urea solubilization buffer

In this method as described by Herman and coworkers [14], soybean seeds were frozen in liquid nitrogen, ground into a fine powder, defatted twice with hexane, and vacuum-dried. Protein was extracted by vortexing 100 mg of seed powder with 1.5 ml of extraction buffer (4% [w/v] CHAPS, 5 M urea, 2 M thiourea, 65 mM DTT, 0.8% [w/v] ampholytes [pH 3–10]) for 5 min at room temperature. The extract was centrifuged at 20,800*g* for 5 min. The supernatant was collected for 2D analysis.

Phenol extraction buffer

This procedure was carried out according to the protocol described by Hurkman and Tanaka [15]. Soybean seed (1 g) was frozen in liquid nitrogen and ground into a fine powder. The powder was extracted in the fume hood by the addition of 2.5 ml of Tris–HCl (pH 8.8) buffered phenol and 2.5 ml of extraction media (0.1 M Tris–HCl [pH 8.8], 10 mM EDTA, 0.4% 2-mercaptoethanol, 0.9 M sucrose). The extract was vortexed for 5 min and sonicated for 30 min at 4 °C. The extract was centrifuged at 20,800*g* for 15 min at 4 °C. The phenol phase was transferred to another tube, and proteins were precipitated by adding 5 volumes of cold (−20 °C) 0.1 M ammonium acetate in 100% methanol. The extract was vortexed and incubated at −20 °C for at least 1 h or overnight. The precipitate was collected by centrifugation (20,800*g*, 20 min, 4 °C), and the pellet was washed twice with 0.1 M ammonium acetate in methanol, with ice-cold 80% acetone, and finally once with cold 70% ethanol. The pellet was resuspended in 0.5 to 1.0 ml of extraction solution (8 M urea, 2 M thiourea, 2% CHAPS, 2% Triton X-100, 50 mM DTT, 0.5% [w/v] ampholytes [pH 3–10]) by pipetting and vortexing at 25 to 30 °C. The samples were incubated for 1 h at room temperature with agitation, and the extract was used for protein determination and 2D analysis.

Modified trichloroacetic acid/acetone precipitation/urea solubilization extraction buffer

This protocol was performed according to Cascardo and coworkers [18] with some modifications. The modifications included a different amount of sample used, a different incubation time, and a different protein resolubilization buffer. For this method, soybean seeds were powdered in liquid nitrogen using mortar and pestle. Soybean seed powder (100 mg) was homogenized with 5 ml of a solution containing 10% (w/v) trichloroacetic acid (TCA) in acetone with 0.07% (v/v)

2-mercaptoethanol. Total protein was precipitated for 1 h or overnight at −20 °C. The extract was centrifuged at 20,800*g* for 20 min at 4 °C. The pellet was washed two or three times with acetone containing 0.07% (v/v) 2-mercaptoethanol. Then the pellet was dried under vacuum for 30 min, and the acetone dry powder was resuspended in 1 ml of lysis buffer (9 M urea, 1% CHAPS, 1% [w/v] ampholytes [pH 3–10], 1% DTT), followed by sonication on ice for 30 min. Insoluble material was removed by centrifugation at 20,800*g* for 20 min at 4 °C, and the supernatant was used in 2D–PAGE analysis.

Protein determination and electrophoresis

The concentrations of proteins extracted by all four methods were determined using the Bradford method [19] and using a commercial dye reagent (Bio-Rad). To determine the protein concentration, all samples were precipitated in 10% (w/v) TCA and resolubilized in 1 N NaOH. We took 100 μg of protein from each extraction method for 2D gel analysis. The first-dimension IEF was performed using 13-cm linear IPG strips (pH 3–10) in the IPGphor system (GE Healthcare). All IPG strips were rehydrated with 250 μl of rehydration buffer (8 M urea, 2% CHAPS, 0.5% ampholytes, 0.002% bromophenol blue) containing 100 μg of protein. The voltage settings for IEF were 500 V for 1 h, 1000 V for 1 h, and 8000 V to a total of 14.5 kVh. The focused strips were either run immediately on a 2D gel electrophoresis or stored at −80 °C. For the 2D gel electrophoresis, the gel strips were incubated with equilibration buffer 1 (50 mM Tris–HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 1% DTT) and equilibration buffer 2 (50 mM Tris–HCl [pH 8.8], 6 M urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, 2.5% iodoacetamide) for 15 min each and subsequently placed onto 12% polyacrylamide gel (18 × 16 cm) with Tris–glycine buffer system as described by Laemmli [20]. Strips were overlayed with agarose sealing solution (0.25 M Tris base, 1.92 M glycine, 1% SDS, 0.5% agarose, 0.002% bromophenol blue). The electrophoresis was performed using the Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare) according to the manufacturer's recommendations. The 2D–PAGE gels were visualized by staining with colloidal Coomassie blue G-250 as described by Newsholme and coworkers [21]. The gels were fixed overnight in 50% ethanol and 3% phosphoric acid, followed by 3 × 30-min washes with distilled water. Then the gels were prestained for 1 h in 34% methanol, 17% ammonium sulfate, and 3% phosphoric acid. Finally, the gels were stained in the same solution containing Coomassie blue G-250 (0.066%) for 2 days. The gels were stored in 20% ammonium sulfate solution and scanned using laser densitometry (PDSI, GE Healthcare). Triplicate samples were used for soybean seed protein extraction and 2D–PAGE analysis.

In-gel digestion of protein spots

Protein spots were excised from the stained gel and washed first with distilled water to remove ammonium sulfate and then with 50% acetonitrile containing 25 mM ammonium bicarbonate to destain the gel plug. The gel plug was dehydrated with 100% acetonitrile, dried under vacuum, and then reswollen with 20 μl of 10 μg/ml trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate. Digestion was performed overnight at 37 °C. The resulting tryptic fragments were extracted with 50% acetonitrile and 5% trifluoroacetic acid with sonication. The extract was dried to completeness and then dissolved in 50% acetonitrile and 0.1% trifluoroacetic acid.

Protein identification

MALDI–TOF–MS analysis of tryptic peptides

A Voyager DE-STR MALDI–TOF mass spectrometer (Applied Biosystems, Framingham, MA, USA) operated in positive ion reflector mode was used to analyze tryptic peptides. Samples were cocrystallized with CHCA matrix, and spectra were acquired with 50 shots of a 337-nm nitrogen laser operating at 20 Hz. Spectra were calibrated using the trypsin autolysis peaks at *m*/*z* 842.51 and 2211.10 as internal standards.

MS/MS analysis of tryptic peptides

A Thermo Finnigan LCQ Deca XP Plus ion trap mass spectrometer was used to analyze proteins that were not positively identified by MALDI–TOF–MS. Peptides were separated on a reverse-phase column using a 30-min gradient of 5 to 60% acetonitrile in water with 0.1% formic acid. The instrument was operated with a duty cycle that acquired MS/MS spectra on the three most abundant ions identified by a survey scan from 300 to 2000 Da. Dynamic exclusion was employed to prevent the continuous analysis of the same ions. Once two MS/MS spectra of any given ion had been acquired, the parent mass was placed on an exclusion list for a duration of 1.5 min. The raw data were processed by Sequest to generate DTA files for database searching. The merge pl script from Matrix Science was used to convert multiple Sequest DTA files into a single mascot generic file suitable for searching in Mascot.

Protein identification was performed by searching the National Center for Biotechnology Information (NCBI) nonredundant database using the Mascot search engine, which uses a probability-based scoring system [22]. The following parameters were used for database searches with MALDI–TOF peptide mass fingerprinting data: monoisotopic mass, 25 ppm mass accuracy, trypsin as digesting enzyme with 1 missed cleavage allowed, carbamidomethylation of cysteine as a fixed modification,

oxidation of methionine, N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modifications. For database searches with MS/MS spectra, the following parameters were used: average mass; 1.5 Da peptide and MS/MS mass tolerance; peptide charge of +1, +2, or +3; trypsin as digesting enzyme with 1 missed cleavage allowed; carbamidomethylation of cysteine as a fixed modification; oxidation of methionine, N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modifications. Taxonomy was limited to green plants for both MALDI and MS/MS ion searches. For MALDI–TOF–MS data to qualify as a positive identification, a protein's score had to equal or exceed the minimum significant score. Positive identification of proteins by MS/MS analysis required a minimum of two unique peptides, with at least one peptide having a significant ion score.

Results and discussion

In this study, we compared four different protein extraction methods with soybean seeds to determine those that increase the solubilization of proteins for subsequent analysis by 2D–PAGE. Because nonprotein impurities can critically affect the quality of 2D–PAGE separation, this study was imperative to evaluate, standardize, and select efficient methods for soybean protein analysis. Among the four methods, urea solubilization (Fig. 1A) and phenol extraction (Fig. 1C) resolved fewer protein spots than did thiourea/urea solubilization (Fig. 1B) and the TCA method (Fig. 1D). In addition, in the urea and phenol methods, protein resolution was poor in several areas and spots were diffuse in the high molecular weight region, particularly in the pH range from 4 to 6. Although overall separations of proteins in the thiourea/urea and TCA methods were quite similar, low molecular weight proteins were consistently more highly resolved when proteins were extracted with the TCA method. These observations support those of Pridmore and coworkers [23] that TCA precipitation enhances resolution of individual protein spots due in part to inhibition of proteolytic activity. We found that the thiourea/urea (Fig. 1B) and TCA/acetone methods (Fig. 1D) enhanced the solubility of total proteins compared with the other two methods. Cascardo and coworkers [18],

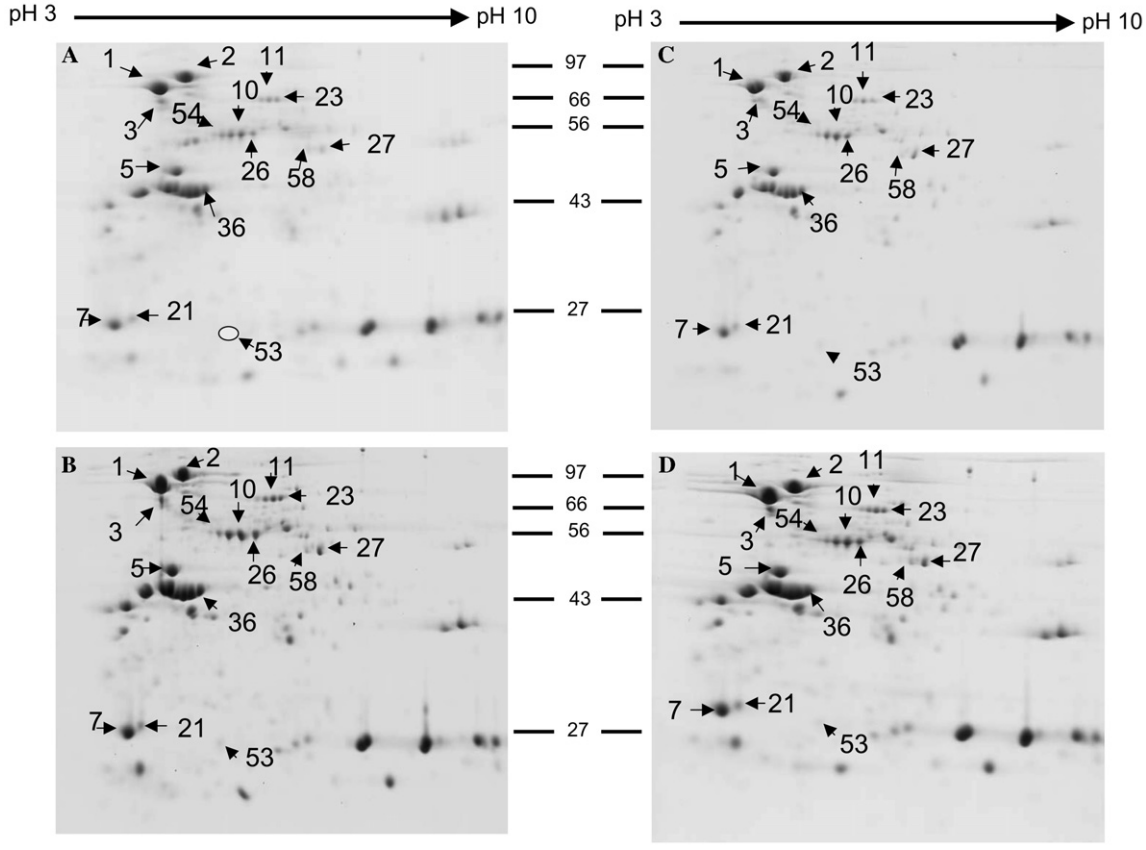

Fig. 1. Two-dimensional electrophoretograms of soybean proteins extracted using various methods: (A) urea solubilization buffer, (B) thiourea/urea solubilization buffer, (C) phenol extraction buffer, and (D) modified TCA/acetone precipitation/urea solubilization extraction buffer. Protein (100 μg) was focused in a rehydration buffer. The first-dimension run used IPG strips (pH 3–10), and the second-dimension run used 12% SDS–PAGE. Gels were stained with colloidal Coomassie blue stain G-250. Arrows indicate the spots showing abundant or less abundant proteins.

Damerval and coworkers [24], and Santoni and coworkers [25] also demonstrated high resolution of proteins by 2D–PAGE using a TCA precipitation method in *Arabidopsis thaliana* and wheat seedlings. Using thiourea/urea and phenol method extractions, we identified proteins similar to those identified by Herman and coworkers [14] and Mooney and Thelen [16]. The efficiency of the TCA method was also tested with soybean cotyledons, cultured root tissues, tobacco flowers, and tobacco leaves (data not shown).

To identify specific proteins following 2D–PAGE, spots were manually picked from colloidal Coomassie blue-stained gels of the TCA method digested with trypsin and analyzed by MS. A total of 15 spots, consisting of both abundant and less abundant proteins recovered from the modified TCA/acetone method, were selected and analyzed to check their quality. The MALDI–TOF–MS and LC–MS showed that all of the excised spots led to good quality spectra, indicating the compatibility of the TCA/acetone method with MS analysis. Data listed in Table 1 include an assigned protein spot number, calculated isoelectric point, molecular weight, protein identity, number of peptide matched, percentage sequence coverage, MOWSE score, expect value, and NCBI database accession number of the best match and databases that yielded concurrent identification.

In soybean seeds, β-conglycinin and glycinin are two major proteins that account for approximately 70 to 80% of the total seed proteins and are responsible for the nutritional, physicochemical, and physiological properties of soybean proteins [26,27]. In our studies, these two major storage proteins, β-conglycinin, and both acidic and basic glycinin polypeptide chains, were well separated using all four extraction procedures (Fig. 1, spots 1, 2, 3, 5, 10, 26, 36, and 54). Relative positions within the gel of these individual abundant proteins were similar across all methods, with minor variations in the intensity. For example, the intensity of β-conglycinin β homotrimer (spots 10, 26, and 54) was weaker and more diffuse when proteins were extracted using the urea (Fig. 1A) or phenol (Fig. 1C) methods compared with extraction using the thiourea/urea (Fig. 1B) and TCA (Fig. 1D) methods. Similar results were seen with glycinin subunits (spots 5 and 36).

Examination of less abundant nonstorage proteins showed differences in intensities depending on the extraction method. Spots 27 and 58 were weak in intensity and diffuse using the urea and phenol extraction methods but were clearly resolved using the thiourea/urea and TCA methods. These spots were not significantly identified by MALDI–TOF–MS but were subsequently identified as alcohol dehydrogenase using LC–MS (Fig. 2A). Likewise, Kunitz trypsin inhibitor, an allergen protein (spots 7 and 21), appeared to be weak using the urea and phenol methods but was clearly resolved using the TCA and thiourea/urea methods. MALDI–TOF–MS analysis revealed a good quality spectrum of proteins (Fig. 2B). In addition, spots 11 and 23, which were identified as sucrose binding protein precursors, were also better resolved using the thiourea/urea and TCA methods. The identity of these proteins by their approximate molecular weights and isoelectric points was similar to that in previous reports [14,28], confirming the reproducibility of our 2D–PAGE analysis. Finally, a less abundant protein (spot 53) was absent using the urea and phenol extraction methods but was present in the protein recovered using the TCA and thiourea/urea methods. This spot was successfully identified as an allergen protein, Gly m Bd 28K, by LC–MS.

Of the four methods we evaluated, the thiourea/urea and TCA methods solubilized approximately the same number of proteins and displayed sharper spot definition in the lower molecular weight range compared with the urea and phenol methods. Based on these results, we found that the modified TCA/acetone and thiourea/urea extraction procedures are efficient methods for recovery of soybean seed proteins for 2D–PAGE analysis. One

Table 1
Proteins identified by MALDI–TOF–MS and LC–MS analyses

Spot ID	Calculated pI/Mr	Protein identity	Peptides matched	Sequence coverage (%)	MOWSE score	Expect Value	NCBI Accession number
1	4.92/63184	α-Subunit of β-conglycinin	25	39	217	3.00E−17	gi|9967357
2	5.23/65160	α′-Subunit of β-conglycinin	20	41	194	5.90E−15	gi|9967361
3	4.92/63184	α-Subunit of β-conglycinin	27	43	250	1.50E−20	gi|9967357
5	5.46/55850	Glycinin A3b4 subunit homohexamer	13	26	96	3.50E−05	gi|33357661
7	4.61/20310	Soybean trypsin inhibitor	15	53	147	3.00E−10	gi|3318877
10	5.67/47879	β-Conglycinin β-homotrimer	25	48	203	7.50E−16	gi|21465628
11	6.42/60884	Sucrose-binding protein precursor	18	34	125	4.70E−08	gi|548900
21	4.97/22817	Kunitz-type trypsin inhibitor precursor	8	42	72	1.10E−02	gi|125722
23	6.42/60884	Sucrose-binding protein precursor	21	45	194	6.00E−15	gi|548900
26	5.67/47879	β-Conglycinin β-homotrimer	26	49	240	1.50E−19	gi|21465628
36	5.78/54047	Soybean proglycinin A1ab1b homotrimer	11	20	139	1.90E−09	gi|15988117
54	5.67/47879	β-Conglycinin β-homotrimer	23	45	219	1.90E−17	gi|21465628
27	6.19/40749	Alcohol dehydrogenase 1	11	17	454		gi|22597178
53	5.73/52813	Allergen Gly m Bd 28K	3	6	187		gi|12697782
58	6.19/40749	Alcohol dehydrogenase 1	7	18	325		gi|22597178

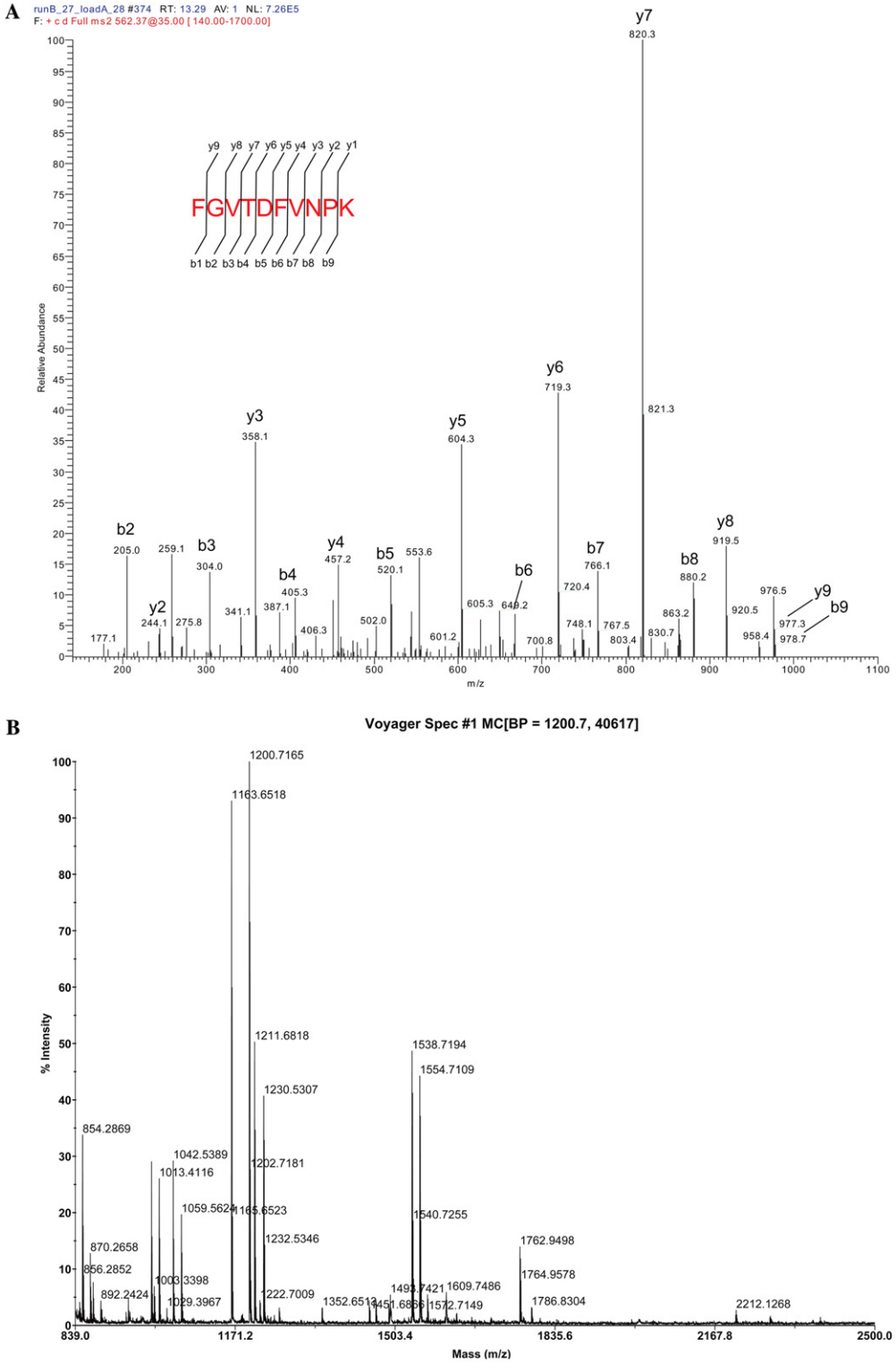

Fig. 2. (A) MS/MS spectrum of one tryptic peptide of spot 27 showing a complete b and y ions series. The protein was identified as alcohol dehydrogenase on the basis of finding seven unique tryptic peptides, each with a significant ion score, and total protein coverage of 17%. (B) MALDI–TOF spectrum of the tryptic digest of spot 7. Of the 37 peaks submitted to the Mascot search engine for database searching, 15 were matched for total sequence coverage of 53%. The overall error rate for the matching peaks was 9 ppm.

possible explanation is that the TCA and thiourea/urea methods remove nonprotein and proteolytic components that interfere with IEF. In addition, analysis of protein spots by both MALDI–TOF and LC–MS demonstrated the compatibility of the TCA method for and identifying soybean seed proteins and subunits. These

tools and methods will be used to study the biosafety of transgenic soybeans in our laboratory.

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